(12) INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(19) World Intellectual Property Organization
International Bureau

(43) International Publication Date
28 February 2002 (28.02.2002)

PCT

(10) International Publication Number
WO 02/16624 A1

(51) International Patent Classification⁷: **C12N 15/82**, 15/11, A01H 5/00, 5/10, C12N 5/10

(21) International Application Number: PCT/SG00/00124

(22) International Filing Date: 25 August 2000 (25.08.2000)

(25) Filing Language: English

(26) Publication Language: English

(71) Applicant *(for all designated States except US)*: **INSTITUTE OF MOLECULAR AGROBIOLOGY** [SG/SG]; 1 Research Link, The National University of Singapore, Singapore 117604 (SG).

(72) Inventors; and
(75) Inventors/Applicants *(for US only)*: **SUNDARESAN, Venkatesan** [US/SG]; 465 Pasir Panjang Road, Singapore 118798 (SG). **HONG, Yan** [SG/SG]; Block 206 Bukit Batok St 21 #06-80, Singapore 650206 (SG).

(74) Agent: MIRANDAH, Gladys; Ella Cheong & G. Mirandah, 111 North Bridge Road, #22-01 Peninsula Plaza, Singapore 179098 (SG).

(81) Designated States *(national)*: AE, AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, CA, CH, CN, CR, CU, CZ, DE, DK, DM, EE, ES, FI, GB, GD, GE, GH, GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MA, MD, MG, MK, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TR, TT, TZ, UA, UG, US, UZ, VN, YU, ZA, ZW.

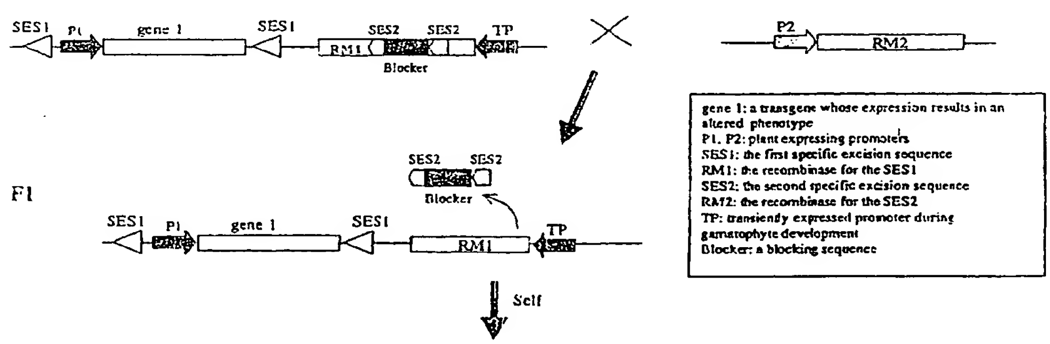
(84) Designated States *(regional)*: European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE).

Published:
— *with international search report*

For two-letter codes and other abbreviations, refer to the "Guidance Notes on Codes and Abbreviations" appearing at the beginning of each regular issue of the PCT Gazette.

(54) Title: REDUCTION OF TRANSMISSION OF TRANSGENES IN PLANTS

Schematic Illustration of the Controlled Excision of a Transgene



(57) Abstract: A method for making a genetically modified plant expressing a transgene with a desirable trait that is transmitted poorly, the trait being lost in ensuing generations due to excision of the transgene. The method involves hybridizing a first plant regenerated from a plant cell that has been transfected with DNA sequences comprising a first gene whose expression results in an altered plant phenotype linked to a promoter, the cassette of gene and promoter flanked on each side by the first specific excision sequences, a second gene that encodes a recombinase specific for the first specific excision sequences linked to a transiently active promoter, the gene and promoter being separated by a blocking sequence flanked on each side by the a second specific excision sequeces to a second plant regenerated from a second plant cell that has been transfected with DNA sequences comprising a third gene that encodes a recombinase specific for the second specific excision sequences linked to a promoter, and growing a hybrid plant from the hybrid seed. Alternatively, a single plant can be stably transformed with all of the above sequences to effect the same result. Plant cells, plant tissues, plant seed and whole plants containing the above DNA sequences are also disclosed.

WO 02/16624 A1

REDUCTION OF TRANSMISSION OF TRANSGENES IN PLANTS

BACKGROUND OF THE INVENTION

The present invention relates to methods for site-specific recombination in plant cells, as well as DNA vectors which can be employed in such methods. More particularly, the invention relates to recombinant DNA vectors containing recombinase-specific sequences and methods for creating a transgenic plant that has been modified such that two site-specific recombination systems may be employed to eliminate one or more controlled utility genes present as a transgene(s). The methods and vectors of the present invention can be used to express one or more transgenes with desirable traits and to prevent transmission of the transgenes to progeny of the plant. Also, expression of the transgenes can be limited to a particular stage of plant development, a particular plant tissue, particular environmental conditions, or a particular time or location, or a combination of these situations. The two site-specific recombination system may be contained in one transgenic plant or may be contained in two transgenic plants that are to be crossed.

Modification of plant varieties is increasingly being used to introduce foreign genes into heterologous host plants in order to confer desirable traits to plants of agricultural and horticultural importance. Some examples are virus resistant tobacco, tomato with a longer shelf life, insect resistant maize and cotton, and herbicide tolerant soybean and canola. With the ever-widening increased application of genetic modification to plants, there is also arising a growing concern for the presence, vertical transmission and lateral spreading of unwanted transgenes (*e.g.* genes which when expressed facilitate selection and/or confer a phenotype that is desired only under certain well-defined conditions) to related weedy plants. Based on the above trends and concerns, there presently exists a need to develop technology to tightly regulate the spread of transgenes following the transformation of plant species with exogenous genes.

Site-specific recombination is the reciprocal genetic exchange between defined sequences present on one or more DNA segments. In site-specific recombination, strand exchange occurs in a conservative manner by the precise breakage and rejoining of the DNA within the site-specific recombination sequences. In general, such reactions are catalyzed by a system-specific recombinase, which in some cases also requires additional factors to facilitate cleavage and excision of insertion of donor DNA sequences. A site specific recombination system has many potential uses for rearranging genetic material in higher eukaryotic cells. Such

a system can operate to invert, insert or delete a targeted DNA sequence positioned between defined site-specific recombination sites located on a DNA molecule.

Many site-specific recombination systems are known which utilize a DNA recombinase that recognizes and cleaves DNA at defined sequences. For example, the bacteriophage P1 Cre/lox site-specific recombination system (Hoess *et al.* 1982; Abremski *et al.* 1983) consists of two components: (i) a recombinase enzyme (Cre) which is the product of the *cre* gene; and (ii) DNA sequence specific recombination sites (*lox*) at which the recombinase acts. The Cre gene encodes a 38 kDa recombinase which, without any other additional factors, catalyzes recombination between two *lox* sites. The 38 kD "Cre" protein product of the bacteriophage P1 *cre* gene is sufficient to catalyze recombination between *lox* sites without any additional co-factors. The nucleotide sequence of the lox site consists of two inverted 13 base pair (bp) repeats separated by an asymmetric 8 bp spacer in which each inverted repeat acts as a binding site for the Cre recombinase. Each inverted repeat and the contiguous 4 bp of the spacer comprise a binding domain for Cre (Hoess *et al.* 1984), and strand exchange occurs at a 6-bp staggered cut within the spacer (Hoess *et al.* 1985).

The asymmetric nature of the 8 bp spacer of the *lox* site gives a directionality to the *lox* site and thereby determines the type of recombination event. It has previously been demonstrated that recombination between two directly repeated *lox* sequences separated by an intervening DNA segment results in the excision of the DNA segment between the sites, whereas recombination between two inverted lox sites produces an inversion of an intervening DNA segment (Abremski *et al.* 1983). In addition, recombination between unlinked lox sites forms a cointegrate molecule. Due to the fact that the Cre/*lox* recombination requires only one gene product and a 34 bp sequence, this system provides a simple means to manipulate DNA in eukaryotic cells.

It has previously been demonstrated that the bacteriophage P1 *cre* gene product can be expressed as a functional recombinase in tobacco (Dale and Ow 1990). Upon expression in tobacco protoplasts, the Cre recombinase recognizes its target *lox* sites and mediates reciprocal genetic crossovers at these sites. As in *E. coli*, the recombination event within plant chromatin is conservative, *i.e.* without loss or alteration of the *lox* sequence or DNA adjacent to the *lox* sequences. The Cre/*lox* system thus provides a useful a tool for manipulating DNA in plant cells without disruption of the DNA sequences flanking the *lox* sites.

Bayley *et al.* (1992) utilized the Cre/*lox* system to exchange gene activity in transgenic plants. The system was used to excise a firefly luciferase gene which had previously been incorporated into the tobacco genome between a functional promoter and a distal hybromycin phosphotransferase (hpt) gene. The presence of the intervening "blocking" luciferase DNA between the promoter and the *hpt* gene prevented the *hpt* gene from being expressed. Deletion of the luciferase blocking DNA resulted in the fusion of a promoter with a distally located coding sequence and the excision resulted in the exchange of luciferase activity for hybromycin resistance of the transgenic plant. This experiment proved the feasibility of activating a gene distal from the promoter by site specific deletion of the intervening sequence with the Cre/*lox* recombinase recombination system.

There are several site-specific recombination systems that have been shown to work in plants in addition to the bacteriophage P1 Cre/lox system and these include: (i) the FLP-FRT system from *Saccharomyces cerevisiae* (O'Gorman et al., 1991); (ii) the GIN/gix system from bacteriophage Mu (Maeser and Kahmann, 1991); (iii) the R/RS system from *Zygosaccharomyces rouxii* (Onouchi *et al.*, 1991); the resolvases (Halls *et al.*, 1993); SSV1 encoded integrase (Muskheekishvili *et al.* 1983; and the maize Ac/Ds transposon system (Shen and Hohn, 1992).

The FLP-FRT recombination system from *Saccharomyces cerevisiae* is based on site-specific recombination mediated by the FLP recombinase at a site-specific recombination target DNA sequence (FRT). The FLP recombinase is encoded by the yeast plasmid 2 um and catalyzes a site specific recombination reaction that results in inversion of a segment of the 2 um plasmid. DNAse protection experiments have shown that the FRT site consists of three 13 bp symmetry elements surrounding an 8 bp core region (Andrews *et al.*, 1985). The FRT site is characterized by three 13 bp repeat elements surrounding an 8-bp core sequence. Two of the repeats are direct repeats located on one side of the core while an additional copy of the repeat is inverted in relation to these two and is located on the other side of the 8 bp core sequence. The FLP protein binds to the symmetry elements and cleaves opposite strands of the DNA at either end of the core, becoming covalently attached to the 3' phosphate and leaving an 8 base overhang with a 5' OH terminus (Schwartz and Sadowski 1989). Jayaram *et al.* (1985) have shown that the "minimal" FLP substrate resides in a 13-bp dyad symmetry element consisting of two flanking repeats surrounding an 8-bp core located within the 65-bp recombination region which contains an additional copy of the 13 bp symmetry element. Kilby *et al.* (1995) have

expressed the FLP recombinase under the regulation of a heat shock promoter using as a target the FRT site containing both the minimal FLP recognition sequence containing two inverted 13 bp repeats separated by the 8 bp core and the additional 13 bp flanking repeat. The presence of the additional 13 bp repeat element enhances the reactivity of the minimal FLP substrate. Moreover, sequences extraneous to the recombination region can also affect the efficiency of the recombination reaction.

The FLP recombinase has been shown to function efficiently in the progeny of crosses made between primary transformed tobacco plants (Lloyd and Davis, 1994). These authors, however, were unsuccessful in their attempts to obtain a transgenic Arabidopsis plant expressing FLP recombinase. By inserting two directionally repeated FRT sites flanking a target gene it is possible, by addition of FLP recombinase, to excise the intervening DNA fragment by site-specific eviction. FLP recombinase mediated excision has also been shown to be reversible, providing means for the introduction of DNA into specific sites in mammalian chromosomes (O'Gorman *et al.*, 1991).

The publications and other materials used herein to illuminate the background of the invention or to provide additional details respecting the practice, are incorporated herein by reference, and for convenience are referenced in the following text and respectively grouped in the appended List of References.

SUMMARY OF THE INVENTION

The present invention is directed to DNA constructs, vectors and methods for using the constructs and vectors to construct a transgenic plant that contains one or more transgenes whose excision can be induced by crossing the plant with plants containing additional factors, by subsequent transformation of the plant with the additional factors, or by providing a single vector containing all of the factors required for controlled excision of the transgene. More particularly, the invention relates to transgenic plants that have been modified such that a transgene that has been introduced into a plant can be excised at various stages of growth and thus limited to a particular stage of plant development, a particular plant tissue, particular environmental conditions, or a particular time or location, or a combination of these situations. By controlling the excision of genes that affect the plant phenotype, it is possible to grow transgenic and hybrid plants where the phenotype is advantageous or desired in one generation

but the phenotype is not desired in subsequent generations. This technique has particular utility in agricultural and horticultural applications.

In one aspect of the invention, a DNA construct is provided which comprises a first and second site-specific recombinase that work in tandem to (1) control activation of the first recombinase and (2) direct the controlled excision of a transgene from a transgenic plant.

In one embodiment, the DNA construct comprises a first DNA sequence comprising a plant-active first promoter operably linked to one or more transgenes whose expression results in an altered plant phenotype and a second DNA sequence comprising a transiently-active second promoter that is active only under specific conditions. The first DNA sequence is flanked by a unique first site-specific recombination sequence that is recognized by a first site-specific recombinase. The transiently active promoter is operably linked to the structural gene for a first site-specific recombinase but the second promoter and first recombinase are separated by a blocking sequence that is in turn bounded at each end by a second and distinct unique site-specific recombination sequence that is recognized by a second site-specific recombinase.

In a second embodiment, the DNA construct comprises the first and second DNA sequences as described in the previous above embodiment and also a third DNA sequence comprising a plant-active third promoter operably linked to the second site-specific recombinase that is specific for the second site-specific recombination sequence. In this embodiment, the plant-active third promoter is preferably a regulatable promoter.

In a second aspect of the invention, vectors containing the DNA constructs are provided.

In a third aspect of the invention, transgenic plant tissue containing the DNA constructs are provided. Plant tissue may be plant cells, plant tissue, plant organs, plants, seeds and the like.

In a fourth aspect of the invention, methods for excising transgenes from transgenic plants are provided.

BRIEF DESCRIPTION OF THE FIGURES

Figure 1 is a Schematic illustration of the system of the present invention wherein the prevention of transmission of a transgene is described.

Figure 2 is a schematic representation of the construction of a NOS blocker sequence flanked by two FRT site-specific recombination sites.

Figure 3 is a schematic representation of the known restriction map of *Arabidopsis* Ecotype Lansburg and a cloning scheme for isolating the atDMC1 promoter region on a plasmid vector for use in the practice of the invention.

Figure 4 is a schematic representation of the construction of an DNA sequence comprising the DNA sequences of *lox*-35S promoter-GUS-NOS3'-*lox*-OCS3"-*bar*-NOS as a contiguous DNA sequence for use in the practice of the present invention.

Figure 5 is a schematic representation showing the assembly of a DNA sequence comprising the atDMC1-FRT-"*nptII*/NOSter"blocker-FRT-Cre-NOS3'sequences as a contiguous DNA sequence for use in the practice of the present invention.

Figure 6 is a schematic representation showing the assembly of a DNA sequence comprising the atDMC1 promoter-FRT-nptII/NOS ter blocker-FRT-Cre-NOS3'-NOS promoter-bar-OCS-*lox*-NOS 3'-GUS-35 S-lox as a contiguous DNA sequence for use in the practice of the present invention.

DETAILED DESCRIPTION OF THE PREFERRED EMBODIMENTS OF THE INVENTION

The present invention involves methods for the construction of a transgenic plant that contains one or more genes whose genetic transmission through reproduction of the plant can be regulated by employing two site-specific recombinase systems. This system achieves a control of gene transmission in subsequent generations that result from the transgenic plant. By controlling the excision of genes that affect the plant phenotype, it is possible to grow transgenic plants where the phenotype is advantageous or desired but the phenotype is not desired in subsequent generations.

In the preferred embodiments of the invention, site-specific recombination sequences are arranged along the DNA molecule to effect excision of the intervening DNA (*e.g.*, as direct repeats of *lox* separated by the intervening DNA). One skilled in the relevant art can readily appreciate, however, that such a system could alternatively be employed in a way such that the recombination between adjacent site-specific recombination sequences could also cause inversion of the intervening DNA if the sequences were arranged in the necessary orientation along the DNA molecule (*e.g.*, as inverted repeats of *lox* separated by the intervening DNA). Accordingly, the term "site-specific excision sequence" when used in the present specification defines a site-specific recombination sequence that is oriented in such a way as to effect removal of the DNA positioned between two such sequences.

A gene that results in an altered plant phenotype is any gene whose expression leads to the plant exhibiting a trait or traits that would distinguish it from a plant of the same species not expressing the gene. Examples of such altered phenotypes include a different growth habit, altered flower or fruit color or quality, premature or late flowering, increased or decreased yield, sterility, mortality, disease susceptibility, altered production of secondary metabolites, or an altered crop quality such as taste or appearance.

Examples of genes useful for expression in transformed plant cells are known in the art. See for example, U.S. Patent No. 5,659,026. Exemplary genes include, but are not limited to, Bt genes or patatin genes for insect resistance; the Hm1 gene and chitinase genes for disease resistance; the pat, bar, EPSP synthase gene or ALS genes for herbicide resistance; genes encoding proteins with altered nutritional properties; genes encoding enzymes involved in starch or oil biosynthetic pathways; down-or up-regulatory sequences for metabolic pathway enzymes; and the like. As those of ordinary skill in the art will recognize, this is only a partial list of possible genes that can be used with the transformation method of the present invention. Furthermore, as those of ordinary skill in the art will also recognize, regulatory sequences including promoters, terminators and the like will also be required, and these are generally known in the art. Zhao et al. (1998) discloses the construction of a prior art superbinary vector pPHP 10525. This vector contains virB, virC and virG genes isolated from superviral strain A281. The vector includes 35Sbar and ubi/GUS plant expression cassettes inserted between the T-DNA borders. Plant expression cassettes preferably comprise a structural gene to which is attached regulatory DNA regions that permit expression of the gene in plant cells. The regulatory regions consist at a minimum of a promoter capable of directing expression of a gene in a plant cell. The promoter is positioned upstream or at the 5' end of the gene to be expressed. A terminator is also provided as a regulatory region in the plant expression cassette and is capable of providing polyadenylation and transcription terminator functions in plant cells. The terminator is attached downstream or at the 3' end of the gene to be expressed. Marker genes, included in the vector, are useful for assessing transformation frequencies in this invention.

One skilled in the relevant art will readily appreciate that any combination of repressible, inducible, and transiently active promoters can be employed with any or all of the DNA sequences contemplated by the present invention (*i.e.*, transgene(s), first, and second recombinases).

As used in this specification, a transiently-active promoter is any promoter that is active either during a particular phase of plant development or under particular environmental conditions, and is essentially inactive at other times. Any appropriate transiently-active promoter can be used, and selection of an appropriate promoter will be governed by such considerations as plant type and the time at which excision of the transgene is desired. The transiently-active promoter is preferably not a "leaky" promoter, meaning that it is active substantially only during a well-defined phase of plant growth or under particular environmental conditions, and substantially inactive at all other times. Such requirements will also be useful for the plant-active promoter controlling the expression of the second recombinase that in certain embodiments will be an inducible promoter. This property prevents the premature "triggering" of the system. There are numerous published examples of transiently-active promoters and inducible promoters which can be applied in the present system, as in, for example, the SPL promoter described in Yang *et al.* (1999). A plant-active promoter is any promoter that is active in cells of a plant of interest. Plant-active promoters can be of viral, bacterial, fungal, animal or plant origin.

A gene and a promoter are to be considered to be operably linked if they are on the same strand of DNA, in the same orientation, and are located relative to one another such that the promoter directs transcription of the gene (i.e. in *cis*). The presence of intervening DNA sequences between the promoter and the gene does not preclude an operable relationship.

The blocking sequence can be any sequence that prevents expression of the gene linked to the transiently-active promotor, such as a termination signal. In those embodiments employing a repressible promoter system, the gene encoding the repressor is responsive to an outside stimulus, or encodes a repressor element that is itself responsive to an outside stimulus, so that repressor function can be controlled by the outside stimulus. The stimulus is preferably one to which the plant is not normally exposed, such as a particular chemical, temperature shock, or osmotic shock. In this way, the simple application of the stimulus will block the repression of the gene it is operably linked to, yet there will be a low probability of the repressor being accidentally or incidentally blocked. If the repressor is sensitive to a chemical stimulus, the chemical is preferably non-toxic to the crop and to non-pest animals. A preferred system is the Tn10 tet repressor system, which is responsive to tetracycline. Gatz, et al. (1992). In this system, a modified Cauliflower Mosaic Virus (CaMV) 35S promoter containing one or more, preferably three, tet operons is used; the Tn10 tet repressor gene produces a repressor protein that binds to the tet operon(s) and prevents the expression of the gene to which the promoter is

linked. The presence of tetracycline inhibits binding of the Tn10 tet repressor to the tet operon(s), allowing free expression of the linked gene. This system is preferred because the stimulus, tetracycline, is not one to which the plant would normally be exposed, so its application can be controlled. Also, since tetracycline has no harmful effects on plants or animals, its presence would not otherwise impede the normal development of the plant, and residual amounts left on the seed or plant after treatment would have no significant environmental impact. The recombinase/excision sequence system can be any one that selectively removes DNA in a plant genome. The excision sequences are preferably unique in the plant, so that unintended cleavage of the plant genome does not occur. Several examples of such systems are discussed in Sauer, U.S. Pat. No. 4,959,317 and in Sadowski (1993). A preferred system is the bacteriophage Cre/*lox* system, wherein the Cre protein performs site-specific recombination of DNA at *lox* sites. Other systems include the resolvases (Hall, 1993), FLP (Pan, et al., 1993), SSV1 encoded integrase (Muskhekishvili, et al., 1993), and the maize Ac/Ds transposon system (Shen and Hohn, 1992).

A site-specific excision sequence is a DNA sequence that is recognized by a site-specific recombinase. A site-recombinase is an enzyme that recognizes a site-specific excision sequence or set of specific excision sequences and effects the removal of, or otherwise alters, DNA between specific excision sequences.

The methods used for the actual transformation of the target plant are not critical to this invention. The transformation of the plant is preferably permanent, e.g. by integration of introduced sequences into the plant genome, so that the introduced sequences are passed onto successive plant generations. There are many plant transformation techniques well-known to workers in the art, and new techniques are continually becoming known. Any technique that is suitable for the target plant can be employed with this invention. For example, the sequences can be introduced in a variety of forms, such as a strand of DNA, in a plasmid, or in an artificial chromosome, to name a few. The introduction of the sequences into the target plant cells can be accomplished by a variety of techniques, as well, such as calcium phosphate-DNA co-precipitation, electroporation, microinjection, *Agrobacterium* infection, liposomes or microprojectile transformation. Those of ordinary skill in the art can refer to the literature for details, and select suitable techniques without undue experimentation. The methods used to regenerate transformed cells into whole plants are not critical to this invention, and any method suitable for the target plant can be employed. The literature describes numerous techniques for

regenerating specific plant types, (e.g., via somatic embryogenesis, Umbeck, et al., 1987) and more are continually becoming known. Those of ordinary skill in the art can refer to the literature for details and select suitable techniques without undue experimentation.

The described example of site-specific recombination involves eviction of an intervening blocking DNA fragment to allow activation of a silent first recombinase and subsequent excision of a transgene. It is also possible to activate genes by site-specific reversion of the intervening DNA fragment when the flanking recombination sites (*lox, e.g.*) are inverted in their orientation relative to each other.

The present invention can be used to make a variety of transgenic plants. The method is particularly suited for use with plants that are planted as a yearly crop from seed. These include, but are not limited to, fiber crops such as cotton and flax; dicotyledonous seed crops such as soybean, sunflower, rapeseeds and peanut; annual ornamental flowers; monocotyledonous grain crops such as maize, wheat and sorghum; leaf crops such as tobacco; vegetable crops such as lettuce, carrot, broccoli, cabbage and cauliflower; and fruit crops such as tomato, zucchini, watermelon, cantaloupe and pumpkin.

Referring now to Figure 1, the transgenic plants of the present invention are prepared by introducing into their genome a series of functionally interrelated DNA sequences, containing several basic elements. A first DNA sequence is provided which comprises a plant-active promoter operably linked to one or more transgenes whose expression results in an altered plant phenotype. This first DNA sequence is flanked by unique first site-specific recombination sequences that are recognized by a first site-specific recombinase. A second DNA sequence is provided which comprises a second plant-active promoter that is active at a particular stage in plant development or under particular environmental conditions ("transiently-active promoter"). This transiently-active promoter is operably linked to a blocking sequence and a distal first site-specific recombinase, wherein the blocking sequence separates the promoter and the first recombinase. The blocking sequence is flanked on each side by a unique second site-specific excision sequence. The second site-specific excision sequences which flank the blocking sequence are recognizable by a second site-specific recombinase, which can bind to the second site-specific excision sequences to direct the removal of the blocking sequence. The blocking sequence prevents transcription of the first recombinase when present and causes expression of the first recombinase when excised.

The methods of the present invention also disclose a third DNA sequence encoding a plant-active promoter operably linked to a second site-specific recombinase which, when expressed, directs the precise excision of the blocking sequence that is flanked on either end by the second-site-specific recombination sequence.

As illustrated in Figure 1, in one embodiment of the invention, a first plant contains the first and second DNA sequences which result in an altered plant phenotype due to expression of the transgenes. DNA containing the third DNA sequence is introduced into a second plant. Without crossing the first to the second plant, the structural gene for the first recombinase is not expressed, even in the stage(s) of the plant life cycle during which the transiently-active promoter is activated. Upon crossing of the two plants, however, the second recombinase is expressed in the resulting hybrid plant and effects removal of the blocking sequence at the second site-specific excision sequences. As a result, the structural gene for the first recombinase specific for the first specific excision sequence is expressed from the transiently-active promoter that is active, for example, in the flowers, prior to or during gametophyte development. In an F1 hybrid plant, the transgene remains expressed and intact until the expression of the first recombinase during gametophyte development in the flowers drives the excision of the DNA sequence of the first gene and promoter flanked by the first specific excision sequences. The excision occurs prior to formation of the pollen and embryo sacs, and hence prevents transmission of the transgene to the developing seeds.

In accordance with a second embodiment of the invention, the third gene that encodes the recombinase specific for the second site-specific excision sequences can also be provided in the same construct with the first and the second DNA sequences of the invention. In this embodiment, the recombinase specific for the second site-specific excision sequences is preferably operably linked to an inducible promoter. When the inducible promoter is expressed, the second recombinase is expressed, followed by excision of the blocker sequence of the second DNA sequence, activation of the first recombinase based on the expression characteristics of the transiently active promoter, and subsequent excision of the transgene(s) of the first DNA sequence.

In accordance with a third embodiment of the invention, the third gene that encodes the recombinase specific for the second specific excision sequences can be introduced by re-transformation of a plant that has already been transformed with the first and second DNA sequences of the invention. In this embodiment, a construct with the third DNA sequence of the

invention operably linked to a plant-active promoter is introduced into a background where the first and second sequences of the invention have already been incorporated into the plant genome. When the third DNA sequence is expressed, the second recombinase is activated, followed by excision of the blocker sequence of the second DNA sequence, activation of the first recombinase based on the expression characteristics of the transiently active promoter, and subsequent excision of the transgene(s) of the first DNA sequence.

The present invention is described by reference to the following examples, which are offered by way of illustration and are not intended to limit the invention in any manner. Standard techniques well known in the art or the techniques specifically described below are utilized in the practice of this invention.

EXAMPLE 1

This example demonstrates the isolation and mutagenesis of the atDMC1 promoter. Referring now to Figure 3, there is shown the known restriction map of *Arabidopsis* Ecotype Lansburg and a cloning scheme for isolating the atDMC1 promoter region. The atDMC1 promoter described in the present invention can be generated using standard molecular genetic cloning and Polymerase Chain Reaction (PCR) techniques that are known to those skilled in the art. Genomic DNA is isolated from Arabidopsis Ecotype Lansburg and PCR-amplified with the primers DMC1(5'-gttaacaccgtttatatgagacaaaatcagctatg-3'; Seq ID No.1) and DMC4 (5'-catccccacttgcgaattcactacc-3'; Seq ID No.2). The resulting product is then digested with restriction enzymes H*ind*III and E*co*RI to yield a H*ind*III/ E*co*RI DNA fragment. The genomic DNA is then PCR-amplified with primers DMC3 (5'-ggtagtgaattcgcaagtggggatg-3'; Seq ID No. 3) and DMC2 (5'-tgctctagactcgctctaagactctctaagctaggaagagtgagag-3'; Seq ID No. 4) and the resulting product is digested with E*co*RI and X*ba*I to yield a E*co*RI /X*ba*I DNA fragment. The two amplified and restriction enzyme digested fragments are then ligated to H*ind*III/X*ba*I digested plasmid vector pMUCBS to generate a atDMC1 promoter with flanking H*ind*III/X*ba*I sites and an internal E*co*RI site. The resulting plasmid with the atDMC1 promoter was verified by DNA sequence analysis of both strands of the insert DNA.

EXAMPLE 2

This example demonstrates the construction of a NOS blocker sequence flanked by two FRT sites. Referring now to Figure 2, there is shown a schematic representation of the cloning process which can be utilized to obtain the blocking sequence flanked on either side by an FRT

site. The FRT sites of this example contain both the minimal FLP recognition sequence and the additional flanking repeat to ensure high efficiency of recombination. Small letters in the FRT sequences represent restriction enzyme specific sequences while capital letters indicate the FRT nucleotides. The plasmid pMUCBS (this is pMUC9 with pBS+(KS) polylinker; Jones, J. *et al.*, 1985) is restriction enzyme digested with B*am*HI and P*st*I and ligated to the FRT-3/FRT-4 sequences of SEQ ID No. 5. The FRT-1, FRT-2, FRT-3, and FRT-4 sequences depicted in the Figures are synthetic oligolucleotides that were synthesized using standard techniques as well known within the art. The resulting DNA is then digested with X*ho*I, filled in to remove overhangs, digested with P*st*I and ligated to the FRT-1/FRT-2 double strand DNA fragment of SEQ ID No. 6. The resulting plasmid is then digested with P*st*I and ligated with a P*st*I fragment containing the nos terminator and nptII gene from plasmid pBI121(Clontech). The resulting plasmid contains the *nptII*/NOS ter blocker flanked by FRT sites (represented by solid arrows). This DNA sequence can be removed and operably linked to a plant-active promoter utilizing the remaining restriction enzyme sites as indicated in Figure 2.

EXAMPLE 3

This example demonstrates the construction of an DNA sequence containing the transiently-expressed atDMC1 promoter and Cre gene separated by a blocking sequence that is flanked on either end by FRT site-specific recombination sequences. Referring now to Figure 5, there is shown a schematic representation of the cloning strategy utilized to construct a DNA sequence containing the *Cre* gene under the control of the atDMC1 promoter. The plasmid generated in Example 1 is digested with H*ind*III/X*ba*I. Plasmid pED23 (Dale *et al.*, 1990) containing the 35S promoter operably linked to the *Cre* recombinase gene which is further operably linked at its 3' end to a NOS termination sequence is also digested with H*ind*III/X*ba*I. The two digestion products are then ligated, digested with X*ba*I, and filled in to remove overhangs. The blocker sequence flanked by FRT sites of Example 2 is also digested with S*pe*I, filled in to remove overhangs, and then digested with K*pn*I. The two resulting DNA fragments are then ligated to yield the following DNA fragments in a 5' to 3' orientation: atDMC1 promoter-FRT-*nptII*/NOSter blocker-FRT-*Cre*-NOS3'. The promoter and *Cre* gene are separated by a blocking sequence flanked by FRT site-specific recombination sequences.

EXAMPLE 4

This example demonstrates the construction of a FLP recombinase gene operably linked to the 35S promoter and the NPT II gene, which encodes for kanamycin resistance, operably linked to the NOS promoter. Utilizing standard molecular cloning techniques as in Examples 1-3, the FLP recombinase is operably linked to a plant-active promoter and inserted on a plant compatible DNA vector along with the gene for kanamycin resistance.

EXAMPLE 5

This example demonstrates the construction of a cassette comprising the GUS gene operably linked to the 35S promoter, with the promoter and gene flanked by *lox* sequences. In addition, this DNA sequence also contains the BAR gene operably linked to the "NOS promoter." The designations "NOS" and "OCS" represent 3' transcription termination and processing sequences for the inserted genes that were derived from *Agrobacterium tumefaciens* nopaline synthase (NOS) and Octopine synthase (OCS) gene sequences. Referring now to Figure 4, the *lox* sequences are designated by the open arrows. The schematic illustrates the sequential restriction enzyme digestion and ligations which lead to the final arrangement of the DNA sequence. A *lox* core sequence as shown with ClaI/SacI overhangs is directionally inserted into the polylinker site of plasmid pTML23. Next, a *lox* core sequence with an A*cc*I overhang and an E*co*RV blunt end is inserted into the polylinker site. This results in two directly repeated *lox* sites within the polylinker that are separated by E*co*RI, P*st*I and M*lu*I sites. Plasmid pSLJ512 containing the NOS promoter operably linked to the *bar* gene and the OCS site is digested with P*st*I, filled in to remove overhangs, digested with B*cl*I and ligated with the above *lox* polylinker that has been digested with C*la*I, filled in to remove overhangs, and digested with B*gl*I. The resulting plasmid is digested with X*ho*I and EcoRV and the NOS promoter/*bar*/OCS fragment is ligated with EcoRV/XhoI digested plasmid pSP72. The resulting plasmid is digested with P*st*I/E*co*RI and ligated with the P*st*I/E*co*RI fragment from plasmid pBI121(Clontech) containing the GUS gene operably linked to the 35S promoter to yield the DNA sequence containing the GUS gene operably linked to the 35S promoter, with the promoter and gene flanked by *lox* sequences and the BAR gene operably linked to the NOS promoter.

EXAMPLE 6

This example demonstrates the assembly of the DNA sequence comprising the sequence of the *atDMC1*-FRT-*nptII*/NOSter blocker-FRT-Cre-NOS3'-NOS promoter-BAR-OCS3'-*lox*-GUS-35S-*lox*. Referring now to Figure 6, Plasmid pZP200A (Hajdukiewicz *et al.* 1994)

indicating the left and right borders ("LB" and "RB") of a polylinker cloning site, is digested with S*ma*I and ligated with the GUS gene operably linked to the 35S promoter, with the promoter and gene flanked by *lox* sequences and the BAR gene operably linked to the NOS promoter (from Example 5) that has been digested with X*ho*I and B*gl*II and filled in to remove overhangs to insert the GUS/*bar* DNA fragment into plasmid pPZP200A. The resulting plasmid and the plasmid containing the atDMC1 promoter-FRT-*nptII*/NOSter blocker-FRT-*Cre*-NOS3' sequences (Example 3) are digested with S*al*I and H*ind*III and ligated to yield a plasmid containing the *atDMC1*-FRT-*nptII*/NOSter blocker-FRT-Cre-NOS3'-NOS promoter-BAR-OCS3'-*lox*-GUS-35S-*lox* DNA fragment on a single DNA vector.

EXAMPLE 7

This example demonstrates a method for the Production of whole plants using the two recombinase system. One kind of transgenic tobacco plant can be made using the techniques disclosed in the present invention that contains the first and second DNA sequences of the disclosed invention on a single DNA fragment in the following order:atDMC1 promoter-FRT-nptII/NOS ter blocker-FRT-Cre-NOS3'-NOS promoter-bar-OCS-*lox*-NOS 3'-GUS-35 S-lox. A plant with this construct that actively expresses GUS and BAR proteins is crossed with a plant that contains an DNA sequence with the FLP recombinase gene operably linked to the 35S promoter and the NPTII kanamycin resistance gene operably linked to the NOS promoter. Progeny that contain the complete system will be selected by their resistance to both kanamycin and bastar, expression of GUS, location of Cre under atDMC1 promoter as a result of the deletion of blocking sequence and the presence of all of the other components of the systems by PCR with suitable primers.

EXAMPLE 8

This example describes methods for the evaluation of transformed plants from Example 7 for the subsequent production of seeds with the transgene deleted. The hybrid progeny are allowed to grow, self-fertilize and produce seeds. Seeds are germinated and progeny that contain the complete system are selected by their resistance to both kanamycin and bastar, loss of expression of GUS, transient expression of Cre under the regulation of the atDMC1 promoter, and the loss of the 35S-GUS cassette and the presence of all of the other components of the system. Because all of the aforementioned DNA sequences are known, one skilled in the

relevant art will readily appreciate that their presence and expression can readily be monitored using standard PCR techniques with the appropriate primers. Transgenic rapeseeds plants containing the complete system can be generated in the same way as for tobacco.

While the invention has been disclosed in this patent application by reference to the details of preferred embodiments of the invention, it is to be understood that the disclosure is intended in an illustrative rather than in a limiting sense, as it is contemplated that modifications will readily occur to those skilled in the art, within the spirit of the invention and the scope of the appended claims.

LIST OF REFERENCES

U.S. Patent 5658772 Aug 1997 to Odell *et al.*

U.S. Patent No. 5,659,026 Aug 1997 to Baszcynski *et al.*.

U.S. Patent 5723765 Mar 1998 to Olliver *et al.*

U.S. Patent 5888732 Mar 1999 to Hartley *et al.*

U.S. Patent 5925808 July 1999 to Oliver *et al.*

U.S. Patent 5929301 July 1999 to Baszcynski *et al.*

U.S. Patent 5929307 July 1999 to Hodges *et al.*

U.S. Patent 5977441 Nov 1999 to Oliver et al.

U.S. Patent No. 4,959,317 September, 1990 to Sauer.

Abremski, K. *et al.* (1983). Studies on the properties of P1 site-specific recombination: evidence for topologically unlinked products following recombination. *Cell* 32:1301-1311.

Amin, A.A. *et al.*(1991). Synaptic intermediates promoted by the FLP Recombinase. *J. Mol. Biol.* 214:55-72.

Amin, A.A. *et al.* (1991). Synapsis, Strand Scission, and Strand Exchange Induced by the FLP Recombinase: Analysis with Half-FRT Sites. *Mol. Cell. Biol.* 11:4497-4508.

Andrews, B.J. *et al.* (1985). The FLP recombinase of the 2 micron circle DNA of yeast: interaction with its target sequences. *Cell* 40:795-803.

Bayley, C.C. *et al.* (1992). Exchange of gene activity in transgenic plants catalyzed by the Cre-*lox* site-specific recombination system. *Plant Mol. Biol.* 18:353-361.

Dale E.C. and Ow D.W. (1990). Intra- and intermolecular site-specific recombination in plant cells mediated by bacteriophage P1 recombinase. *Gene* 91:79-85.

Dale E.C. and Ow D.W. (1991). Gene transfer with subsequent removal of the selection gene from the host genome. *Proc. Natl. Acad. Sci. U.S.A.* 88:10588-10562.

Gatz *et al.* (1992). Stringent repression and homogenous de-repression by tetracycline of a modified CaMV 35S promoter in intact transgenic tobacco plants. *Plant J.* 2:397-404.

Hajdukiewicz P., Svab, Z. and Maliga, P. (1994) The small versatile pPZP family of Agrobacterium binary vectors for plant transformation. *Plant Mol.Biol.* 6:989-94.

Hall, Samantha C. and Halford S.E. (1993). Specificity of DNA recognition in the nucleoprotein complex for site-specific recombination by Tn21 resolvase. *Nucleic Acid Res.* 21:5712-5719.

Hoess, R.H., Ziese, M. And Sternberg, N. (1982). P1 Site-specific recombination: nucleotide sequence of the recombining sites. *Proc. Natl. Acad. Sci. U.S.A.* 79:3398-3402.

Hoess, R.H. and Abremski, K. (1984). Interaction of the bacteriophage P1 recombinase Cre with the recombining site *loxP. Proc. Natl. Acad. Sci. U.S.A.* 81:1026-1029.

Hoess, R. and Abremski, K. (1985). Mechanism of strand cleavage and exchange in the Cre-*lox* site-specific recombination system. *J. Mol. Biol.* 181:351-362.

Jayaram, M. (1985). Two-micrometer circle site-specific recombination: the minimal substrate and the possible role of flanking sequences. *Proc. Natl. Acad. Sci. USA* 82:5875-5879.

Jones, J., Dunsmuir, P. and Bedbrook, J. (1985). *EMBO J.* 4:2411-2418.

Kilby, N.J. *et al.* (1995). FLP recombinase in transgenic plants: constitutive activity in stably transformed tobacco and generation of marked cell clones in Arabidopsis. Plant J. 8:637-652.

Lloyd, A.M. and Davis, R.W. (1994). Functional expression of the yeast FLP/FRT site–specific recombination system in *Nicotiana tabacum. Mol. Gen. Genet.* 242:653-657.

Lyznik, L.A. *et al.* (1993). Activity of yeast FLP recombinase in maize and rice protoplasts. *Nucl. Acids Res.* 21,969-975.

Muskhelishvili, Georgi, *et al.* (1993). SSV1-encoded site-specific recombination system in sulfolobus shibate. *Mol. Gen. Genet.* 237:334-342.

Odell, Joan T., *et al.* (Oct. 2, 1994). Seed-Specific Gene Activation Mediated by the Cre/lox Site-Specific Recombination System. *Plant Physiol.*, 106:447-458.

O'Gorman, S. *et al.* (1991). *Science* 251: 1351-1355.

Qin, Minmin, *et al.*, (1994) Cre recombinase-mediated site-specific recombination between plant chromosomes, *Proc. Natl. Acad. Sci. USA* 91:1706-1710 .

Sauer, Brian, Manipulation of Transgenes by Site-Specific Recombination: Use of Cre Recombinase, Methods in Enzymology, 225:890-900 (1993).

Schwartz, C.J.E. and Sadowski P.D. (1989) FLP Recombinase of the 2 um Circle Plasmid of Saccharonyces cerevisiae Bends Its DNA Target: Isolation of FLP mutants defective in DNA bending. J. Mol. Biol. 205:647-658

Shen, W.H. and Hohn, B. (1992) Excision of a transposable element from a viral vector introduced into maize plants by agroinfection. Plant J. 2:35-42

Yang, W.C., Ye, D., Xu, J. and Sundaresan, V. (1999) The Sporocyteless gene of Arabidopsis is required for initiation of sporogenesis and encodes a novel nuclear protein. Genes and Develop. 13:2108-2117.

Zhao, Z.Y. et al. (1998). International Published Patent Application No. WO 98/32326.

CLAIMS

1. A method for excising from a plant at a specific time a first DNA sequence encoding at least one transgene whose expression results in an altered plant phenotype comprising:

stably transforming a plant cell or culture with the first DNA sequence flanked on each side by a first site-specific excision sequence;

stably transforming the plant cell or culture with a second DNA sequence having a gene that encodes a first recombinase specific for the first site-specific excision sequence operably linked to a transiently active promoter, the gene and promoter of the second DNA sequence being in functional relation to one another, but separated by a blocking sequence that is flanked on each side by a second site-specific excision sequence, such that the presence of the blocking sequence prevents the expression of the first recombinase and excision of the blocking sequence causes the transiently-active promoter to regulate the expression of the first recombinase;

regenerating the cell or cell culture into a first transgenic plant; and

providing the first transgenic plant with a third DNA sequence having a gene that encodes a second recombinase specific for the second site-specific excision sequence operably linked to a second plant-active promoter.

2. A DNA construct comprising:

a first DNA sequence encoding at least one transgene whose expression results in an altered plant phenotype operably linked to a plant active promoter, wherein the first DNA sequence is flanked on each side by a first site-specific excision sequence;

a second DNA sequence having a gene that encodes a first recombinase specific for the first site-specific excision sequence operably linked to a transiently active promoter, the gene and promoter of the second DNA sequence being in functional relation to one another, but separated by a blocking sequence that is flanked on each side by a second site-specific excision sequence, such that the presence of the blocking sequence prevents the expression of the first recombinase and excision of the blocking sequence causes the transiently-active promoter to regulate the expression of the first recombinase; and

a third DNA sequence having a gene that encodes a second recombinase specific for the second site-specific excision sequence operably linked to a second plant-active promoter.

3. - A DNA construct comprising:

a first DNA sequence encoding at least one transgene whose expression results in an altered plant phenotype operably linked to a plant active promoter, wherein the first DNA sequence is flanked on each side by a first site-specific excision sequence; and

a second DNA sequence having a gene that encodes a first recombinase specific for the first site-specific excision sequence operably linked to a transiently active promoter, the gene and promoter of the second DNA sequence being in functional relation to one another, but separated by a blocking sequence that is flanked on each side by a second site-specific excision sequence, such that the presence of the blocking sequence prevents the expression of the first recombinase and excision of the blocking sequence causes the transiently-active promoter to regulate the expression of the first recombinase.

4. A DNA vector containing a DNA construct of claim 2 or claim 3 further comprising at least one selectable marker permitting stable transformation and selection of host cells containing the DNA construct.

5. A method according to claim 1 wherein:

the transiently-active promotor is selected from the group consisting of a promotor active in late embryogenesis, in seed development, in flower development, in leaf development, in root development, in vascular tissue development, in pollen development, after wounding, during heat or cold stress, during water stress, or during or after exposure to heavy metals;

the first and second plant-active promoters are selected from the group consisting of constitutive and transiently-active promoters and are the same or different from each other;

the first and second site-specific excision sequence are selected from the group consisting of *lox* sequences and sequences recognizable by either flippase, resolvase, FLP, SSV1-encoded integrase, or transposase; and

the first and second site-specific recombinase are selected from the group consisting of CRE, flippase, resolvase, FLP, SSV1-encoded integrase, and transposase.

6. A method according to claim 1 wherein:

a second transgenic plant cell or culture is stably transformed with the third DNA sequence;

the second transgenic plant cell or culture is regenerated into a second transgenic plant;

the second transgenic plant is hybridized with the first transgenic plant to produce hybrid seeds containing the first, second, and third DNA sequences; and

the hybrid seed is grown to produce a hybrid plant.

7. A method according to claim 1 wherein plant cells or culture from the first transgenic plant are stably transformed with the third DNA sequence and the cell or cell culture is regenerated into a plant.

8. A method according to claim 1 wherein the plant cell or cell culture is simultaneously stably transformed with the first, second and third DNA sequences and the second recombinase specific for the second site-specific excision sequence is operably linked to an inducible promoter.

9. A transgenic plant comprising:

a first DNA sequence having at least one gene whose expression results in an altered plant phenotype operably linked to a first plant-active promoter, the first DNA sequence flanked on each side by a first site-specific excision sequence;

a second DNA sequence having a gene that encodes a first recombinase specific for the first site-specific excision sequence operably linked to a transiently active promoter, the gene and promoter of the second DNA sequence being in functional relation to one another, but separated by a blocking sequence that is flanked on each side

by a second site-specific excision sequence, such that the presence of the blocking sequence prevents the expression of the first recombinase and excision of the blocking sequence causes the transiently-active promoter to regulate the expression of the first recombinase; and

a third DNA sequence having a gene that encodes a second recombinase specific for the second site-specific excision sequence operably linked to a second plant-active promoter.

10. A transgenic plant comprising:

a first DNA sequence having at least one gene whose expression results in an altered plant phenotype operably linked to a first plant-active promoter, the first DNA sequence flanked on each side by a first site-specific excision sequence; and

a second DNA sequence having a gene that encodes a first recombinase specific for the first site-specific excision sequence operably linked to a transiently active promoter, the gene and promoter of the second DNA sequence being in functional relation to one another, but separated by a blocking sequence that is flanked on each side by a second site-specific excision sequence, such that the presence of the blocking sequence prevents the expression of the first recombinase and excision of the blocking sequence causes the transiently-active promoter to regulate the expression of the first recombinase.

11. A transgenic plant according to claim 9 or claim 10 wherein:

the transiently-active promotor is selected from the group consisting of a promotor active in late embryogenesis, in seed development, in flower development, in leaf development, in root development, in vascular tissue development, in pollen development, after wounding, during heat or cold stress, during water stress, or during or after exposure to heavy metals;

the first plant-active promoter is selected from the group consisting of constitutive and transiently-active promoters and are the same or different from each other;

the first and second site-specific excision sequence are selected from the group consisting of *lox* sequences and sequences recognizable by either flippase, resolvase, FLP, SSV1-encoded integrase, or transposase; and

the first and second site-specific recombinase are selected from the group consisting of Cre, flippase, resolvase, FLP, SSV1-encoded integrase, and transposase.

12. A transgenic plant according to claim 9 or claim 10, wherein the transiently active promoter is selected from the group of promoters that are active during sporogenesis or gametophyte development such as the SPL promoter or the atDMC1 promoter.

13. A transgenic plant according to claim 9 or claim 10, wherein the transiently active promoter is the promoter for atDMC1.

14. A transgenic plant according to claim 9 or claim 10, wherein the transiently-active promoter is the SPL promoter.

15. A transgenic plant according to claim 9 or claim 10 wherein the transiently active promoter is replaced with a constitutively active plant promoter.

16. A transgenic plant according to claim 9 or claim 10 wherein the second plant-active promoter is an inducible promoter.

17. A transgenic plant according to claim 9 or claim 10, wherein the first site-specific excision sequence is *lox*, the first recombinase is Cre, the second site-specific excision sequence is FRT, and the second recombinase is FLP.

18. A transgenic plant according to claim 9 or claim 10, wherein the first site-specific excision sequence is FRT, the first recombinase is FLP, the second site-specific excision sequence is *lox*, and the second recombinase is Cre.

19. A plant seed comprising:

a first DNA sequence having at least one gene whose expression results in an altered plant phenotype operably linked to a first plant-active promoter, the first DNA sequence flanked on each side by a first site-specific excision sequence;

a second DNA sequence having a gene that encodes a first recombinase specific for the first site-specific excision sequence operably linked to a transiently active promoter, the gene and promoter of the second DNA sequence being in functional relation to one another, but separated by a blocking sequence that is flanked on each side by a second site-specific excision sequence, such that the presence of the blocking sequence prevents the expression of the first recombinase and excision of the blocking sequence causes the transiently-active promoter to regulate the expression of the first recombinase; and

a third DNA sequence having a gene that encodes a second recombinase specific for the second site-specific excision sequence operably linked to a second plant-active promoter.

20. A plant seed comprising:

a first DNA sequence having at least one gene whose expression results in an altered plant phenotype operably linked to a first plant-active promoter, the first DNA sequence flanked on each side by a first site-specific excision sequence; and

a second DNA sequence having a gene that encodes a first recombinase specific for the first site-specific excision sequence operably linked to a transiently active promoter, the gene and promoter of the second DNA sequence being in functional relation to one another, but separated by a blocking sequence that is flanked on each side by a second site-specific excision sequence, such that the presence of the blocking sequence prevents the expression of the first recombinase and excision of the blocking sequence causes the transiently-active promoter to regulate the expression of the first recombinase.

21. A plant seed according to claim 15 or claim 16 wherein:

the transiently-active promotor is selected from the group consisting of a promotor active in late embryogenesis, in seed development, in flower development, in

leaf development, in root development, in vascular tissue development, in pollen development, after wounding, during heat or cold stress, during water stress, or during or after exposure to heavy metals;

the first plant-active promoter is selected from the group consisting of constitutive and transiently-active promoters and are the same or different from each other;

the first and second site-specific excision sequence are selected from the group consisting of *lox* sequences and sequences recognizable by either flippase, resolvase, FLP, SSV1-encoded integrase, or transposase; and

the first and second site-specific recombinase are selected from the group consisting of Cre, flippase, resolvase, FLP, SSV1-encoded integrase, and transposase.

22. A plant seed according to claim 19 or claim 20, wherein the transiently active promoter is selected from the group of promoters that are active during sporogenesis or gametophyte development such as the SPL promoter or the atDMC1 promoter.

23. A plant seed according to claim 19 or claim 20, wherein the transiently active promoter is the promoter for atDMC1.

24. A plant seed according to claim 19 or claim 20, wherein the transiently-active promoter is the SPL promoter.

25. A plant seed according to claim 19 or claim 20 wherein the transiently active promoter is replaced with a constitutively active plant promoter.

26. A plant seed according to claim 19 or claim 20 wherein the second plant-active promoter is an inducible promoter.

27. A plant seed according to claim 19 or claim 20, wherein the first site-specific excision sequence is *lox*, the first recombinase is Cre, the second site-specific excision sequence is FRT, and the second recombinase is FLP.

28. A plant seed according to claim 19 or claim 20, wherein the first site-specific excision sequence is FRT, the first recombinase is FLP, the second site-specific excision sequence is *lox*, and the second recombinase is Cre.

29. Plant tissue comprising:

a first DNA sequence having at least one gene whose expression results in an altered plant phenotype operably linked to a first plant-active promoter, the first DNA sequence flanked on each side by a first site-specific excision sequence;

a second DNA sequence having a gene that encodes a first recombinase specific for the first site-specific excision sequence operably linked to a transiently active promoter, the gene and promoter of the second DNA sequence being in functional relation to one another, but separated by a blocking sequence that is flanked on each side by a second site-specific excision sequence, such that the presence of the blocking sequence prevents the expression of the first recombinase and excision of the blocking sequence causes the transiently-active promoter to regulate the expression of the first recombinase; and

a third DNA sequence having a gene that encodes a second recombinase specific for the second site-specific excision sequence operably linked to a second plant-active promoter.

30. Plant tissue comprising:

a first DNA sequence having at least one gene whose expression results in an altered plant phenotype operably linked to a first plant-active promoter, the first DNA sequence flanked on each side by a first site-specific excision sequence; and

a second DNA sequence having a gene that encodes a first recombinase specific for the first site-specific excision sequence operably linked to a transiently active promoter, the gene and promoter of the second DNA sequence being in functional relation to one another, but separated by a blocking sequence that is flanked on each side by a second site-specific excision sequence, such that the presence of the blocking sequence prevents the expression of the first recombinase and excision of the blocking sequence causes the transiently-active promoter to regulate the expression of the first recombinase.

31. Plant tissue according to claim 29 or claim 30 wherein:

the transiently-active promotor is selected from the group consisting of a promotor active in late embryogenesis, in seed development, in flower development, in leaf development, in root development, in vascular tissue development, in pollen development, after wounding, during heat or cold stress, during water stress, or during or after exposure to heavy metals;

the first plant-active promoter is selected from the group consisting of constitutive and transiently-active promoters and are the same or different from each other;

the first and second site-specific excision sequence are selected from the group consisting of *lox* sequences and sequences recognizable by either flippase, resolvase, FLP, SSV1-encoded integrase, or transposase; and

the first and second site-specific recombinase are selected from the group consisting of Cre, flippase, resolvase, FLP, SSV1-encoded integrase, and transposase.

32. Plant tissue according to claim 29 or claim 30, wherein the transiently active promoter is selected from the group of promoters that are active during sporogenesis or gametophyte development such as the SPL promoter or the atDMC1 promoter.

33. Plant tissue according to claim 29 or claim 30, wherein the transiently active promoter is the promoter for atDMC1.

34. Plant tissue according to claim 29 or claim 30, wherein the transiently-active promoter is the SPL promoter .

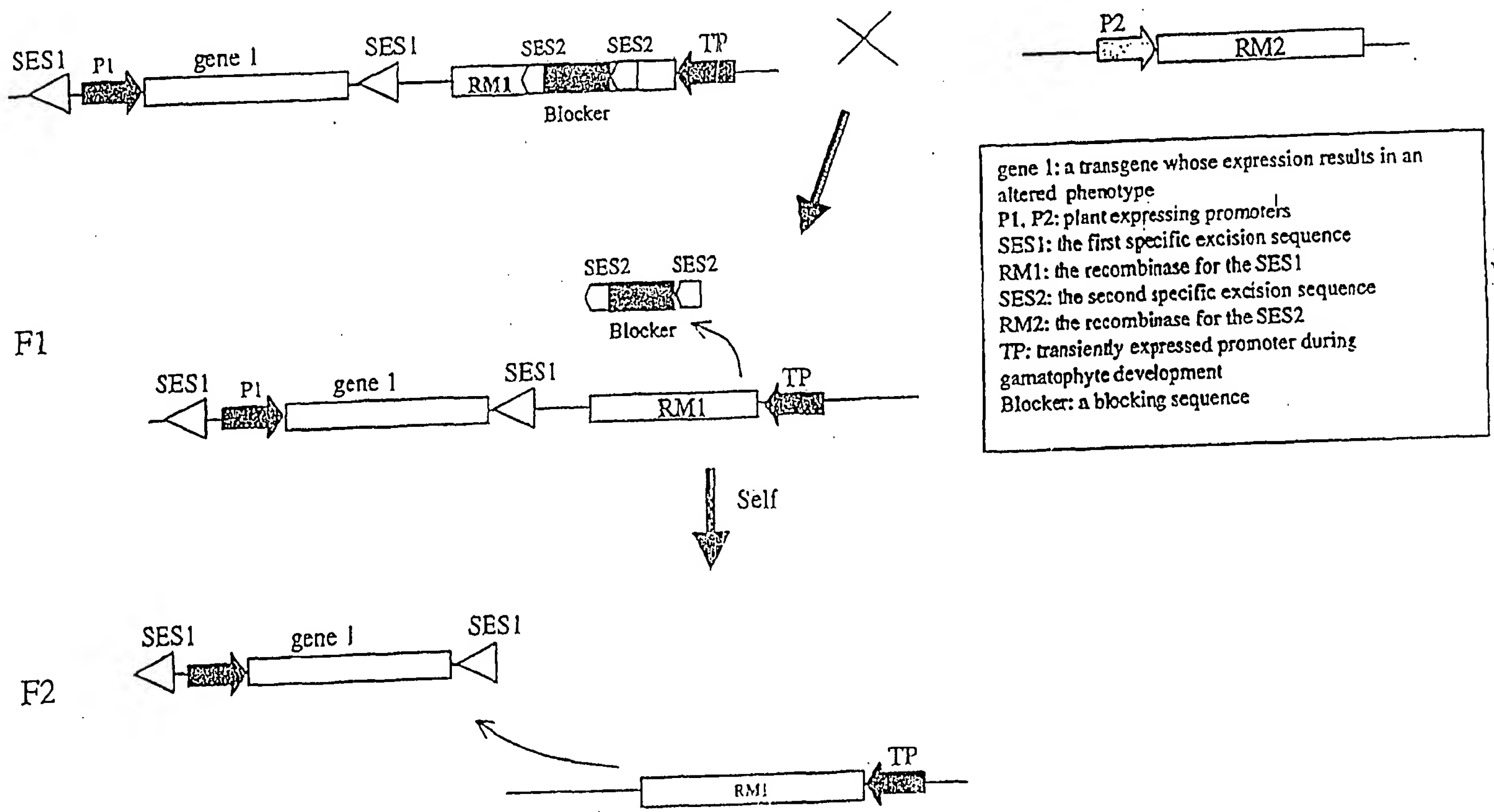
35. Plant tissue according to claim 29 or claim 30 wherein the transiently active promoter is replaced with a constitutively active plant promoter.

36. Plant tissue according to claim 29 or claim 30 wherein the second plant-active promoter is an inducible promoter.

37. Plant tissue according to claim 29 or claim 30, wherein the first site-specific excision sequence is *lox*, the first recombinase is Cre, the second site-specific excision sequence is FRT, and the second recombinase is FLP.

38. Plant tissue according to claim 29 or claim 30, wherein the first site-specific excision sequence is FRT, the first recombinase is FLP, the second site-specific excision sequence is-*lox*, and the second recombinase is Cre.

Fig. 1 Schematic Illustration of the Controlled Excision of a Transgene



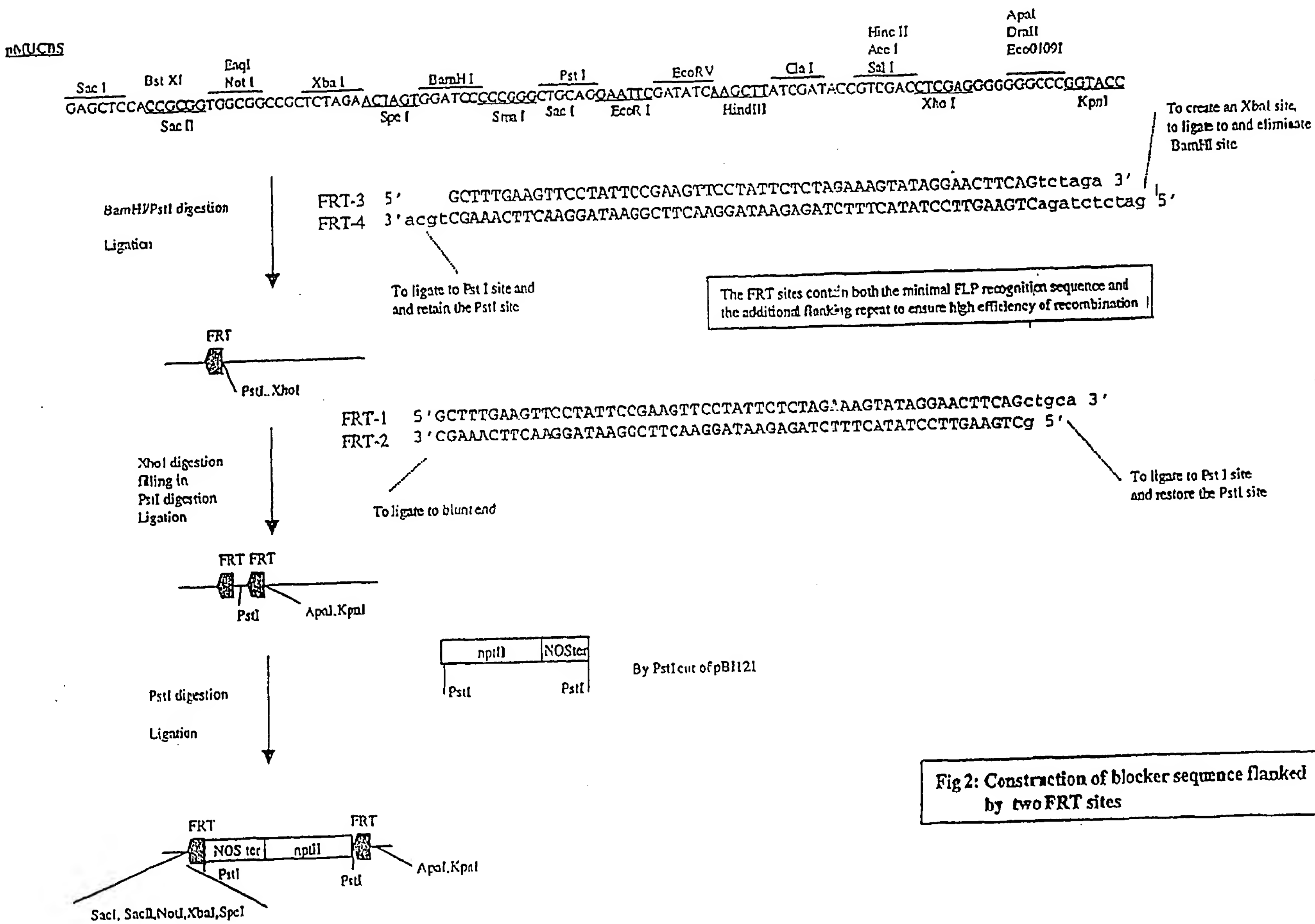


Fig 2: Construction of blocker sequence flanked by two FRT sites

Restriction mapping of atDMC1 promoter region (Klimyuk and Jones JDG 1997):

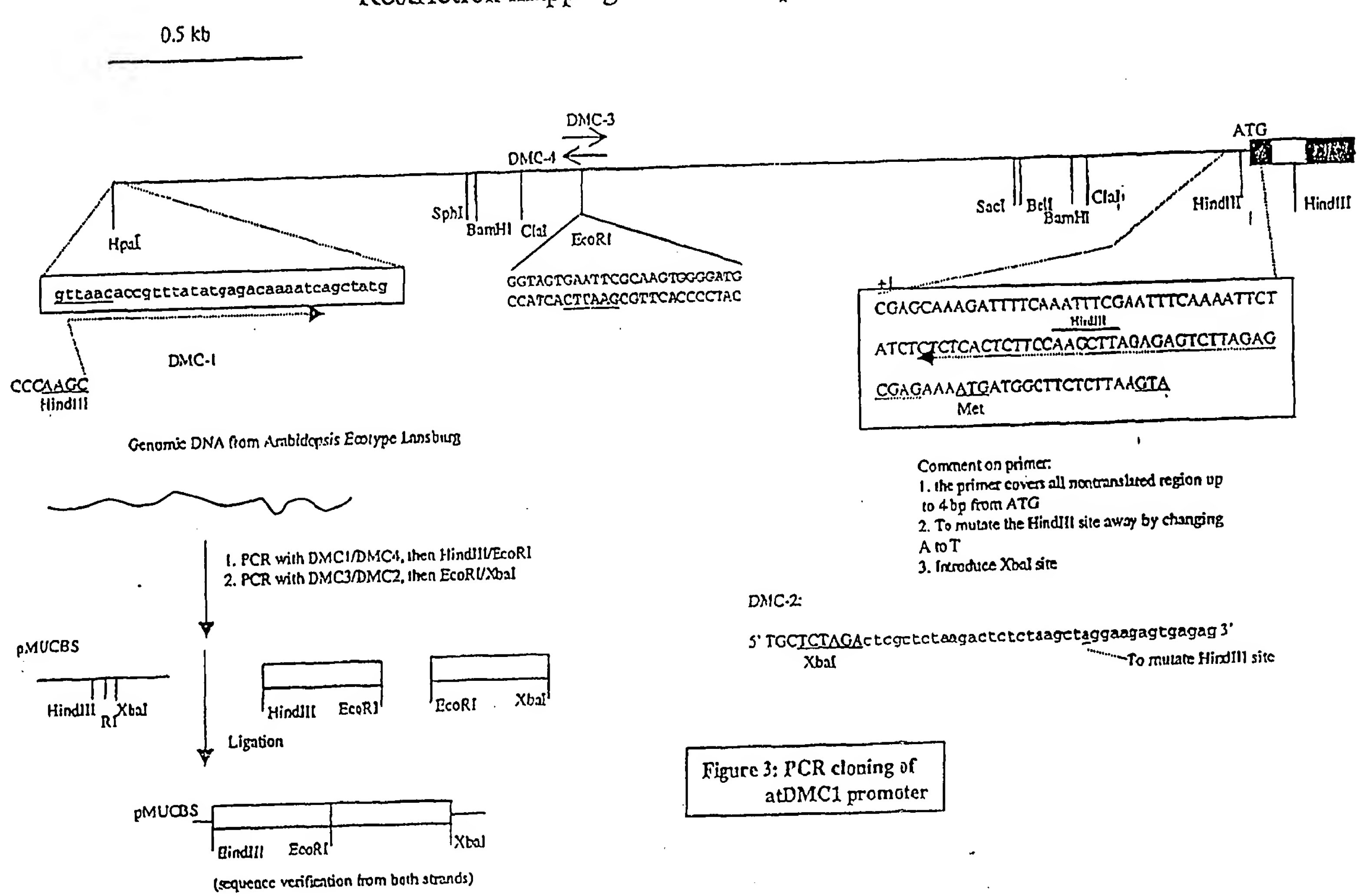


Figure 3: PCR cloning of atDMC1 promoter

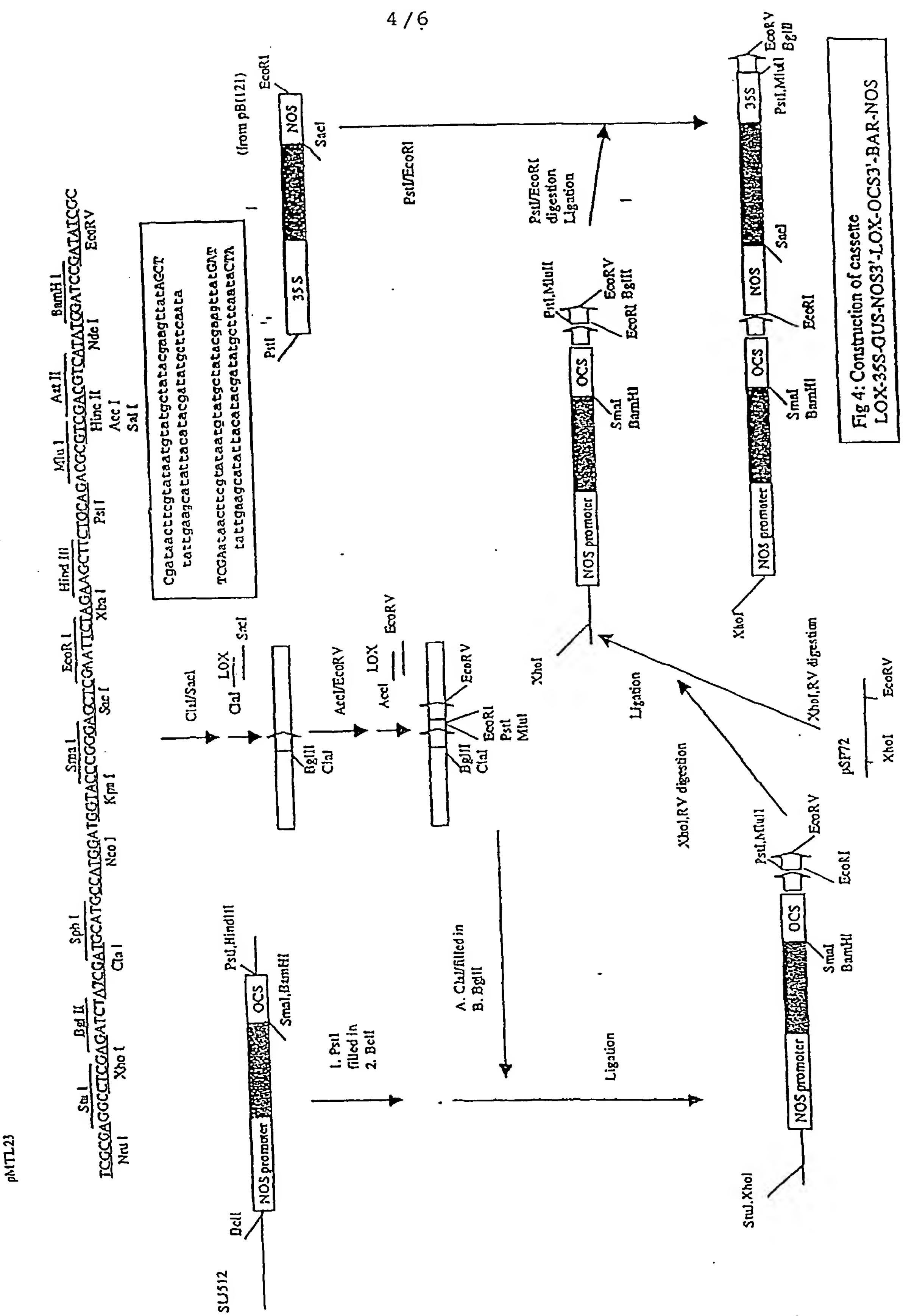


Fig 4: Construction of cassette
LOX-35S-GUS-NOS3'-LOX-OCS3'-BAR-NOS

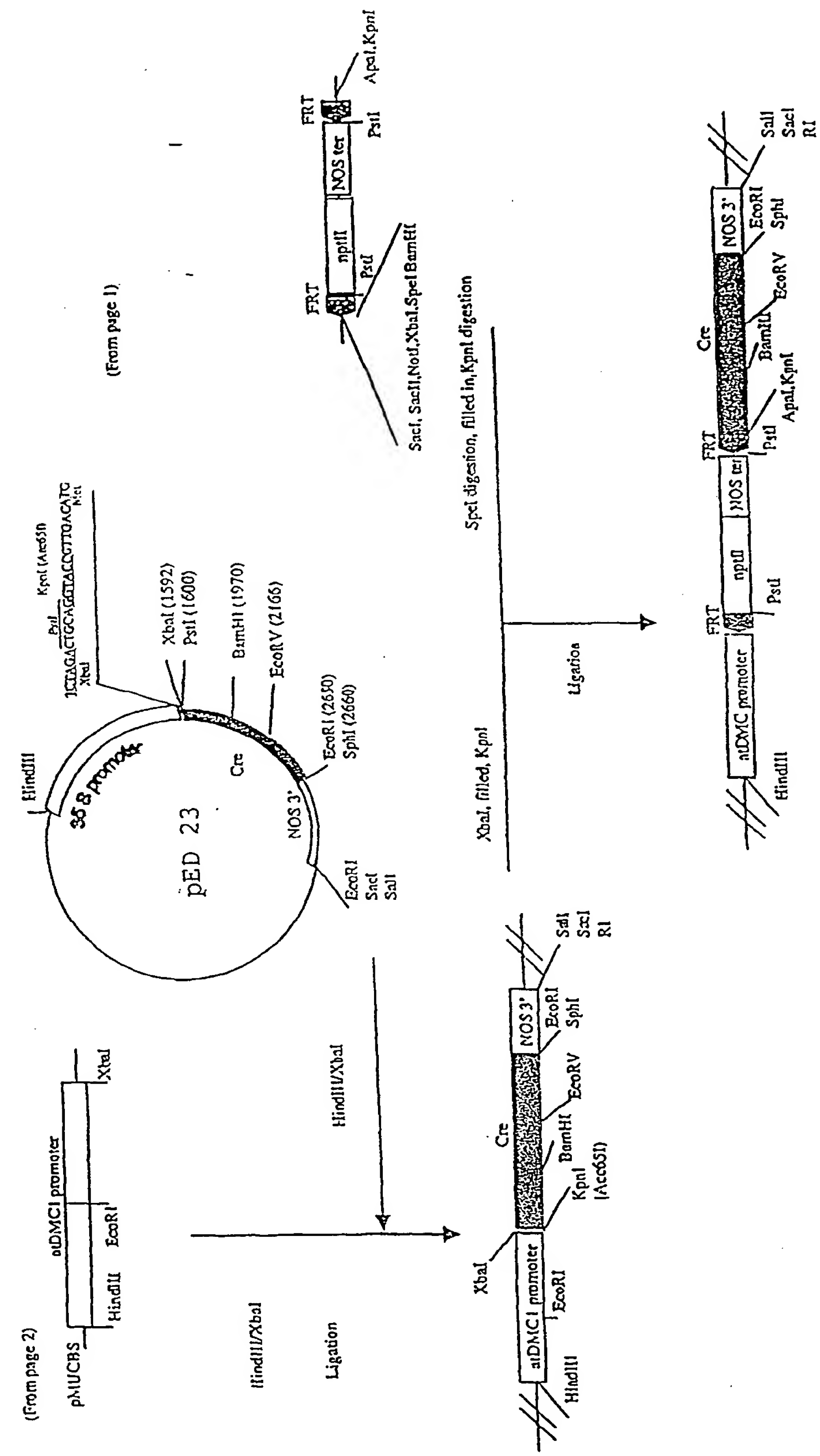


Figure 5. atDMC1 promoter-FRT-blocker-FRT-Cre

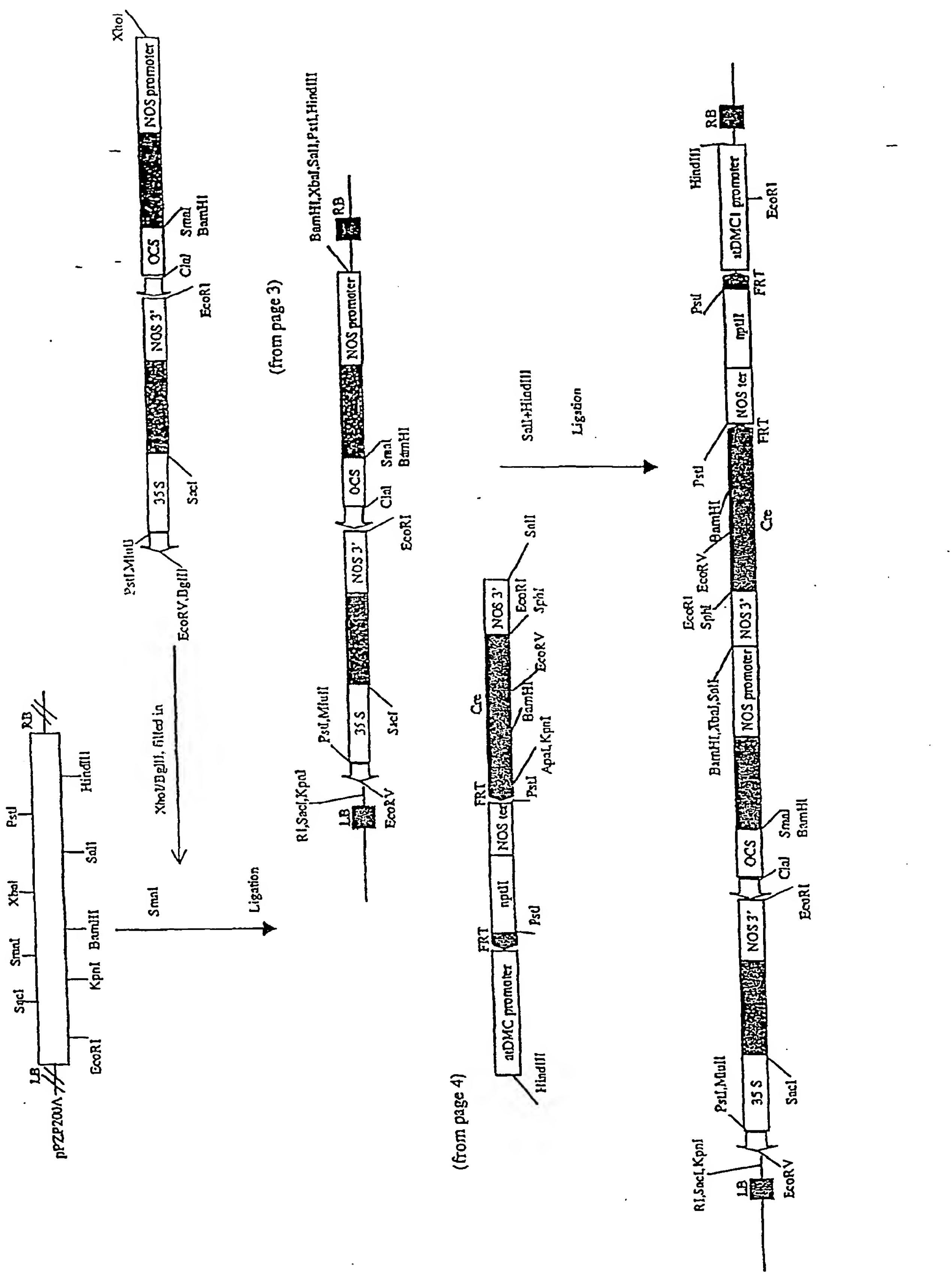


Fig. 6 Final Assembly of atDMC1-FRT-blocker-FRT-CRE-NOS3'-NOS-BAR-OCS3'-LOX-GUS-35S-LOX

INTERNATIONAL SEARCH REPORT

International Application No
PCT/SG 00/00124

A. CLASSIFICATION OF SUBJECT MATTER
IPC 7 C12N15/82 C12N15/11 A01H5/00 A01H5/10 C12N5/10

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 7 C12N

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

WPI Data, EPO-Internal, MEDLINE, BIOSIS

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO 99 10488 A (WAHL GEOFFREY ;GORMAN STEPHEN O (US); SALK INST FOR BIOLOGICAL STU) 4 March 1999 (1999-03-04) page 5, line 25 -page 13, line 27; examples 6-9	1-38
X	WO 99 25841 A (BASZCZYNSKI CHRISTOPHER L ;GORDON KAMM WILLIAM J (US); PIONEER HI) 27 May 1999 (1999-05-27) page 16, line 1 -page 18, line 27	1-38
X	WO 99 23234 A (DUN CORNELIS MARIA PETRUS VAN ;MOGEN INT (NL); GODDIJN OSCAR JOHAN) 14 May 1999 (1999-05-14) page 7, paragraph 3 -page 8, paragraph 1; claim 4	1-38

-/--

[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

° Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
11 April 2001	19/04/2001
Name and mailing address of the ISA European Patent Office, P.B. 5818 Patentlaan 2 NL – 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax: (+31-70) 340-3016	Authorized officer Bilang, J

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication,where appropriate, of the relevant passages	Relevant to claim No.
X	WO 96 04393 A (US AGRICULTURE ;DELTA & PINE LAND CO (US)) 15 February 1996 (1996-02-15) page 3, line 16-33	1-38
X	WO 99 11807 A (SNYDER JOHN R ;HODGES THOMAS K (US); LYZNIK LESZEK A (US); PURDUE) 11 March 1999 (1999-03-11) the whole document	1-38
X	KILBY N ET AL: "FLP recombinase in transgenic plants: constitutive activity in stably transformed tobacco and generation of marked cell clones in Arabidopsis" PLANT JOURNAL,GB,BLACKWELL SCIENTIFIC PUBLICATIONS, OXFORD, vol. 8, no. 5, 1 November 1995 (1995-11-01), pages 637-652, XP002089983 ISSN: 0960-7412 abstract; figures 2,3	1-38
A	WO 93 01283 A (US AGRICULTURE) 21 January 1993 (1993-01-21) page 6, line 7 -page 7, line 23	

INTERNATIONAL SEARCH REPORT

ɔrmation on patent family members

Inte nal Application No
PC / SG 00/00124

Patent document cited in search report		Publication date	Patent family member(s)			Publication date
WO 9910488	A	04-03-1999	AU	9209098	A	16-03-1999
WO 9925841	A	27-05-1999	US	5929301	A	27-07-1999
			AU	1463899	A	07-06-1999
			EP	1045911	A	25-10-2000
			US	6175058	B	16-01-2001
WO 9923234	A	14-05-1999	AU	1487799	A	24-05-1999
			BR	9813340	A	22-08-2000
			CN	1280626	T	17-01-2001
			EP	1027449	A	16-08-2000
			ZA	9809782	A	04-05-1999
WO 9604393	A	15-02-1996	US	5723765	A	03-03-1998
			AU	696668	B	17-09-1998
			AU	3205095	A	04-03-1996
			BR	9508471	A	28-10-1997
			CA	2196410	A	15-02-1996
			EP	0775212	A	28-05-1997
			JP	10503377	T	31-03-1998
			TR	960448	A	21-07-1996
			US	5977441	A	02-11-1999
			US	5925808	A	20-07-1999
			ZA	9506410	A	11-03-1996
WO 9911807	A	11-03-1999	AU	9221098	A	22-03-1999
			EP	1009843	A	21-06-2000
WO 9301283	A	21-01-1993	CA	2073412	A	09-01-1993